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REDUCTION OF MICROSOMIAL PHOSPHOLIPIDS IN EXPERIMENTAL CARDIAC DISCOMPENSATION. (HEART-LUNG OF RAT PREPARATION)

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Reduction of Microsomial Phospholipids in Experimental Cardiac Discompensation. (Heart-Lung of Rat Preparation)*

by

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SUMMARY. — Because previous results had shown that experimental heart failure was not caused by reduction of oxidable substrate and high-energy phosphate content in myocardial tissue, further experiments have been performed in order to see if any alteration in subcellular membranes, which are strictly involved in the excitation-contraction coupling mechanism, could be detected in failing heart.

Experimental cardiac failure was realized by imposing a high load on the left ventricle of the rat heart-lung preparation. The determination of mitochondrial and microsomal phospholipid classes has shown: (1) no change in mitochondrial phospholipids and (2) a 20% reduction in phosphatidylcholine, 35% in phosphatidylserine and 34% in sphingomyelin in the microsomal fraction from failed heart. These biochemical changes could also elucidate the impaired functional activity of sarcoplasmic reticulum from failing heart observed "in viro" by several Authors.

* One part of this work was communicated to the 22nd Congress of the Italian Society of Physiology in Pavia, September, 1970 (31).

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The results of preceding research on cardiac failure, done on heart-lung of rat preparation (21-24), had indicated that the cause of the contractile deficit could be attributed to a diminished capacity to utilize energy on the part of the myocardium, which would be connected to alterations in the liberation-captation processes of Ca^{++} at the level of the myofibrillular apparatus.

In these processes the recent literature recognizes an important part played in the sarcoplasmatic network. The experiments of Legato and Langer (18), which demonstrates in fact, a higher and specific concentration of a Ca^{++} in the terminal sacs of functioning cardiac muscle, suggest that Ca^{++} in the myofilamentment region be released from such structures to the extent which seems to occur in skeletal muscles (42,43). Moreover, in the presence of ATP, the sarcoplasmatic reticule, isolated from myocardial homogenates (vesicular fragments), is able to remove Ca^{++} from the incubator (7,16,14,28), lowering the concentration to levels compatible with the release of the "live" cardiac fiber (15,41).

The importance of the phospholipid component in relation to the function of the sarcoplasmatic reticule appears notable: according to Carvalho (4), in fact, lecithin and phosphatidylserine are probably the phospholipids most directly involved in the properties of cationic exchange in such membranes. Martonosi et al. (19) and Ebashi and Yamanouchi (6) have demonstrated besides that the treatment of microsomes of skeletal muscle with C phospholipase, which determines the degradation of about 90% of the existing lecithin (19), inhibits the ATP-ase activity and the transport capacity of the Ca^{++} of the reticular membranes. Such properties are then renewed with the addition of the mycellular dispersions of synthetic phospholipids (19).

In this research we wanted to examine the possibility that the reduction of the contractile function of the heart at the start of the first signs of discompensation, is due not so much to a shortage of energy (32, 33, 38), necessary for the "calcium pump," as to a reduction of the phospholipid quantity involved in its operation.

Experimental Section

The research was done on Wistar rats with an average weight of 200 g.; the animals, kept without food for nine hours before the experiment, were submitted to light ether narcosis (30) to set the stage for the heart-lung preparation made up according to the technique already described elsewhere (21).

The mytochondrial and microsomial phospholipids were distinguished in hearts lifted in the course of two types of experiment:

1) heart-lung preparations brought to a state of discompensation through the imposition of a highter hemodynamic load (cardiac load = 36 ml/min.; average arterial pressure = 150 mm Hg). In this group of preparations, the heart was taken out at the first signs of discompensation, which announced themselves, after about 20 minutes of activity at the predetermined himodynamic regimen, by the rapid and irreversible fall of the cardiac load (discompensation of maximum strength, Wmax);

2) heart-lung preparations on which a moderate hemodynamic load was imposed (cardiac load = 36 ml/min; average arterial pressure = 90 mm Hg), so that the heart would work in conditions analogous to the physiologic ones of the whole animal. The activity of this group of preparations (control), always perfectly balanced, was interrupted after a period of time equal to the average survival time of the discompensated preparations.

At the end of the duration of the experiment the heart was quickly moved again and immersed in a solution at 0° containing KCl 120 mM and L (--) histidine 5mM. at pH 7.2. The ventricular tissue, carefully dried and released from the aorta and the atriums, was subsequently cut in small pieces and homogenized by hand for 15 minutes at 0° in an homogenizer of the Potter-Elvehjem type with a Teflon piston, containing 4 ml of KCl 120 mM and L (--) histidine 5 mM at pH 7.2 solution. The homogenate was centrifugalized at 500 X g for 10 minutes at 4°; this low gravity was sufficient for a complete sedimentation of erythrocytes, cellular detritus, nuclei and myofibrils. That which remained floating at the top was then centrifugalized at 3000 X g for 30 minutes at 4°; the heavy mytochondrial quantity thus obtained was re-suspended at halfway of the original volume of KCl 120 mM and L (--) histidine 5 mM solution and resedimented in the same conditions.

The top-floating substance resulting from the bath, combined with that obtained from the first centrifugation at 300 X g. was centrifugalized at 8000 X g for 10 minutes at 4°. The small amount of sediment obtained, containing light mytochondroids and a few microsomial membranes, was separated, and the top-floating substance, finally, was centrifugalized at 28000 X g for 60 minutes at 4°. The microsomes thus obtained were suspended in KCl and L (--) histidine solution and resedimented at the same conditions.

The examination at the electronic microscope of the microsomial quantity demonstrated that it was comprised solely of systems of membranes organized in vesicles, realistically recognizable as elements of the sarcoplasmatic reticule. The same examination of the sediment relative to the mytochondroids indicated that such "organules" were for the most part sufficiently well-preserved, especially in regard to the membranes both external and internal. The few membranes

occasionally observed in the mytochondrial sediment probably derive from the occasional breaking of a mytochondroid.

The mytochondrial and microsomial quantities were resuspended to a final concentration of proteins equal to .5—.7 mg/ml.

Aliquots of such particlized suspensions, after an addition of .5 vol. of 3 M trichloroacidic acid (27), were agitated at room temperature for 10 min. The precipitate obtained was carefully washed and resuspended in .25 ml of bidistilled water; after the addition of 2.5 ml of methanol containing HCl .1 N (3), the samples were agitated at 55° for 15 min.; 5 ml of chloroform and a further agitation for 15 min. at room temperature completed the extraction.

The chloroform stage was eliminated under a reduced pressure, at 23° in an azote environment, using a turning evaporator. On the remaining watery phase the extraction of lipids according to the manner described above were repeated. The final chloroform extract was subsequently washed with KCl 0.88% (8); after centrifugalization at 300 X g for 2 hours, the chloroform phase containing the lipids was transferred into a graduated cylinder.

Aliquots of the extract thus obtained were utilized partly for the direct determination of the total phospholipids in the two subcellular quantities (2) and partly for the separation of the neutral lipids from the phospholipids with silicic acid according to Abramson and Blecher (1).

The different classes of phospholipids were separated through chromatography on a fine stratum with a mixture of chloroform: methanol: hydratized ammonium 4 N (65:30:5), using chromatoplates of silicon gel which had been previously activated (.25 mm density). The lipidic areas, localized with iodine vapors, were circumscribed and removed. Their phospholipid content was then

directly determined, without elution from the silicon, measuring the phosphate chlorometrically after a complete oxidation of the organic substance present (2).

The proteins were determined according to the method of Minari and Zilversmit (20), using bovine seralbumin as the standard; a factor of 6.25 was used for the conversion of the azote into protein.

As a test of significance "Student's t" was used, posing p = .05 as a limit of significance.

Experimental Results

In Table I, the total phopolipid content of the mytochondrial and microsomial fractions of control rat hearts and rat hearts after discompensation, is reported, being measured on the basis of the total lipidic extract before the separation of neutral lipids through chromotography on silicic acid and before the separation of the phospholipids in individual classes.

The average content of phospholipids (expressed as phospholipidic phosphorous per mg of protein) of the two subcellular fractions taken in examination in control hearts, shows itself to be consistently higher in the microsomial fraction (24.88 'mu' g P/mg prot.), in accordance with what has been found by other authors (11, 12).

It is noted, however, that, for both fractions, the values of the total phospholipidic phosphorous obtained by us in the controls (Table I) are different from those reported in the literature (12). However, with regard to these, the serious objection is posed that the method for determining the protein content of the subcellular fractions must be considered inadequate either because of the interference due to the saccharose (9,26,34), or because of the lack of sufficient

solubilization of the membrane proteins with de-ossiculated (deoxidized) sodium (36); these drawbacks don't condition the method of determining proteins which we adopted (20).

The results presented in Table I show also that experimental cardiac failure determines a notable reduction (of about 30%) of the total phospholipidic content of the microsomial fraction, whereas it causes no variation in the level of mitochondrial phospholipids.

Table I

The content of phospholipids of the mytochondrial and microsomial fractions of the rat myocardium during balanced work and in discompensation. The determination of the phosphorous (P) (phospholipidic) was done directly on the lipidic extract before the separation of the neutral lipids and the chromatographic separation into individual phospholipidic classes. In parenthesis is indicated the number of experiments. Average ± e.s.

a. Condizioni sperimentali	b. Fosfolipidi totali	
	c. Mitocondri µg P/mg prot.	d. Microsomi µg P/mg prot.
e. Controlli	14,94 ± 0,85 (10)	24,88 ± 2,11 (10)
f. Scompenso W_{max}	15,08 ± 1,08 (9)	17,31 ± 2,04 * (9)

*Significant differences with regard to the control.

a. Experimental conditions
b. Total phospholipids
c. Mytochondroids 'mu' P/mg protein
d. Microsomes 'mu' P/mg protein
e. Control
f. Discompensated W_{max}

The division in phospholipidic classes after the separation of the neutral lipids and the subsequent chromatographic separation on a fine stratum (Table II), besides confirming the statistically significant reduction of the microsomial

content in the total phospholipids (valued as the sum of the individual classes taken in the test), tells us that the reduction is particularly evident, especially in phosphatidylcoline, sphingomyelin and phosphatidylserine, whereas in the case of phosphatidylethanolamine, it does not reach the established level of significance.

Table III reports the result of a similar analysis conducted on the mytochondrial phospholipidic fraction. Both in the total content obtained as a sum of the individual classes and in the individual classes, it is not possible to observe any statistically significant variation between control hearts and discompensated hearts.

It is interesting to find, finally, that the value of the total phospholipids considered as the sum of the individual classes taken in the test (Tables II and III) is lower than the corresponding value obtained when making the determination on the lipidic extract before the separation of the neutral lipids and the separation of the individual phospholipidic classes (Table I). This is almost certainly due to the notable complexity of the analytic operations adopted in these two final phases of the method of determination and to the scarcity of the material available to carry out the determinations, wherein any loss, even if quantitatively minimal, weighed heavily on the possibility of a final advantageous recovery.

In every case, however, both with regard to the mytochondrial and the microsomial fractions of control hearts and hearts after discompensation, this recovery, while it was still reduced, maintained itself in very constant values.

Table II

Content of individual phospholipidic classes in the microsomial fraction of the myocardium of rat during compensated work and in discompensation. The number of experiments is indicated in parentheses. Averate ± e.s.

a. Condizioni sperimentali	b. PL tot. µg P/mg prot.	c. PE µg P/mg prot.	d. PC µg P/mg prot.	e. SP µg P/mg prot.	f. PS µg P/mg prot.
g. Controlli	9,70 ± 0,71 (6)	1,52 ± 0,19 (6)	4,08 ± 0,26 (6)	2,54 ± 0,29 (6)	1,54 ± 0,07 (6)
h. Scompenso W_{max}	7,16 ± 0,52 * (7)	1,20 ± 0,15 (7)	3,27 ± 0,23 * (7)	1,68 ± 0,21 * (7)	1,00 ± 0,12 * (7)

Abbreviations used: PE = phosphatidylethanolamine; PC = phosphatidylcoline; SP = sphingomyline; PS = phosphatildylserine; PL tot. = the total sum of the phospholipids obtained from the sum of the individual classes.

*Significant differences with respect to the control.

a. Experimental condition
b. PL tot.
c. PE
d. PC
e. SP
f. PS
g. Control
h. Discompensated W_{max}

Table III

Content of the individual classes of phospholipids in the mytochondrial fraction of the rat myocardium during balanced work and in discompensation. The number of experiments is indicated in parentheses. Average ± e.s.

a. Condizioni sperimentali	b. PL tot. µg P/mg prot.	c. PE µg P/mg prot.	d. PC µg P/mg prot.	e. SP µg P/mg prot.	f. PS µg P/mg prot.
g. Controlli	5,96 ± 0,56 (6)	1,18 ± 0,22 (6)	2,54 ± 0,24 (6)	1,26 ± 0,16 (6)	0,98 ± 0,08 (6)
h. Scompenso W_{max}	5,70 ± 0,38 (5)	1,27 ± 0,20 (5)	2,17 ± 0,11 (5)	1,30 ± 0,11 (5)	0,96 ± 0,09 (5)

Abbreviations used: PE = phosphatidylethanolamine; PC = phosphatidylcoline; SP = sphyngomyline; PS = phosphatidylserine; PL tot. = the total sum of the phospholipids obtained from the sum of the individual classes.

a. Experimental condition
b. PL tot.
c. PE
d. PC
e. SP
f. PS
g. Control
h. Discompensated W_{max}

Discussion

The results obtained in this research put in evidence a notable reduction (of about 30%) of the phospholipidic content of the sarcoplasmatic reticule coinciding with the first signs of cardiac failure.

In the complex succession of events which characterize the excitement-contraction coupling action (5) and in particular in the processes of captation-liberation of Ca^{++}, the sarcoplasmatic reticule develops, as is well noted, an important role for which the intervention of its phospholipidic components seems to be essential. This is suggested, in fact, by the experiments of Martonosi et al. (19) which show how the treatment of microsomes with C phospholipase and the subsequent degradation of 90% of the lecithin present in such membranes, inhibits the ATP-ase activity and the carrying capacity of Ca^{++}.

The reduction of the microsomial phospholipidic content, which we demonstrated at the occurrence of the first signs of discompensation, appears therefore to have a particular bearing and is also in accord with some functional alterations of the sarcoplasmatic reticule, observed, still in cardiac failure, by other authors. Gertz et al. (10), in fact, working on the sarcoplasmatic reticule isolated from heart lung preparations of dog which were discompensated through the imposition of a higher hemodynamic regimen, were able to demonstrate a reduction in the velocity of captation of Ca^{++} (by 27%) and of the ATP-ase activity; Suko et al. (40) came to analogous results in their work on the sarcoplasmatic reticule of the discompensated right ventricle of calf, and more recently, Schwartz et al. (35) on that of the hamster. It is possible that in the sarcoplasmatic reticule of discompensated hearts, the reduction of the phospholipidic content observed in the present research has connections with the reduction of the ATP-ase activity and of the carrying capacity of the Ca^{++} noted in analogous conditions by the authors cited above.

To evaluate the influence that the diminution of the individual phospholipids examined could have in determining some functional alterations of the sarcoplasmatic reticule coinciding with discompensation, it would be necessary to know exactly the capacity to bind the Ca^{++} of each micosomial phospholipidic component, according to the type of analysis recently confronted by Hess et al. (13). In any case, the reduction we observed in the comparison of lecithin and phosphatidylserine could be of notable importance; it is noted, in fact, that such phospholipids are able to bind several mono- and bi-valent cations, among which is Ca^{++} (4). In case one of their diminutions in the terminal sacs should be verified, that is in that portion of the sarcoplasmatic reticule which "in vivo" is the seat of accumulation and release of Ca^{++} in the various phases of the contractile cycle (18, 37), a lesser release of Ca^{++} towards the region of the myofilaments, either absolutely or in a given span of time, could be derived. What would follow would therefore be a diminution of the strength as well as the velocity of contraction in the muscle, as Spann et al. have found in effect in the strength-velocity curve of the papillary muscle (discompensated) of cat (39).

The relationship we hypothesized between the diminution of the microsomial phospholipidic content and alteration in the captation-liberation processes of Ca^{++} at the level of the myofibrillar apparatus in cardiac failure, besides indicating a possible cause of the reduced contractile capacity of discompensated myocardium, could justify that which in previous research we had observed in relation to the action of adrenalin on cardiac failure. In conditions of a higher hemodynamic load, in fact, the addition, repeated in time, of physiologic doses of adrenalin tripled survival and the work supplied by the heart, delaying discompensation (29). The content in total phospholipids in the fresh ventricular

tissue remained normal during the entire period of balance and diminished significantly only with the appearance of the first signs of failure.

It could therefore reasonably be supposed that the adrenalin can influence the phospholipidic metabolism of the myocardium, thus contributing to the functional integrity of the sarcoplasmatic reticule. In support of such possibilities are the results obtained by Kunze and Olthoff (17) which, analagous to that already observed by Nakatani et al. (25) in the isolated rat aorta, have demonstrated that adrenalin determines an increase in the incorporation of ^{32}P in the principal phospholipids of the myocardium.

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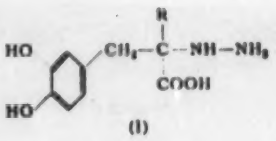
PATENTS

Phenylproprionic derivates involved in cerebral activity, Bayne, G.M., Merck & Co., Inc., (German patent published 1.949.376, June 18, 1970).

The acids L-alpha-hydrazine-3,4-diossiphenylpropionic (I) in which R = H or methyl:



(I)

their racemates and their salts, are used "in therapy" in combination with L- or DL-dopa in a 1:.2-8 relation, for oral administration against dopamine deficiency in the brain. (Chem. Abs., 73, 48546s; 1970).

Spasmolytic compositions against bronchitis. Davies, E.G., Duches M., Johnston, P.T., Imperial Chemical Industries Ltd. (German patent 1.946.387, Sept. 10, 1970).

Spasmolithic compositions are prepared from an amine-like adrenalin or isoprenaline and an s-tiazol-(1.5-a)-pyrimidine derative.

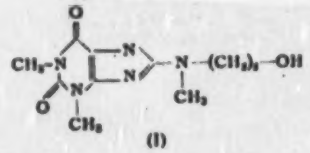
For example, the 2-amino-6-methyl-4-propil-4.5-dyhydro-s-triazol-(1.5-a)-pyrimidin-5(4H) and 2-(third butylamide)-1-(4-oxi-3-(oximethyl)phenyl) ethanol are combined with amide of wheat, $Ca_3(PO_4)_2$ and stearated in Mg and compressed in presses.

This and similar compositions can even be used as aerosol-spray and serve to cure bronchial spasms.

(Chem. Abs., 74, 15726t; 1971).

Composition against the cold containing a derivate of caffein. Klosa, J.; Pape, J.; Arzneimittelwerk Fisher, O.H.G., (German patent 1.918.758, Nov. 5, 1970).

The composition against the cold contains 20-100 mg of 8-(N-methyl-N-(beta-oxiethyl)amino) caffein (I):



(I)

1-10 mg of N-methyl-4-(benzidryloxi)-piperidine and 100-500 mg of Na (anti-pyrinylmethylamino)methansulphonated.

It serves, besides for the treatment of the cold, for curing catarrh of an allergic and infective origin.

(Chem. Abs., 74, 15722p; 1971).